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(54) Title: MODIFIED G-PROTEIN COUPLED RECEPTORS

(57) Abstract

Modified G-protein coupled receptors having deletions in the third intracellular domain are identified and methods of making the modified receptors are provided. The invention includes the modified receptors, assays employing the modified receptors, cells expressing the modified receptors, and compounds identified through the use of the modified receptors, including modulators of the receptors.

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TITLE OF THE INVENTION

MODIFIED G-PROTEIN COUPLED RECEPTORS

CROSS RELATED TO OTHER APPLICATIONS

This is a continuation-in-part of U.S. Serial Number 08/267,987, filed June 29, 1994, now pending, and an continuation-in-part of U. S. Serial Number 08/267,987, filed June 29, 1994, now pending.

BACKGROUND OF THE INVENTION

G-protein coupled receptors are cell surface receptors that mediate the responses of the cell to a variety of environmental signals. Upon binding an agonist, the receptor interacts with one or more specific G proteins, which then regulate the activities of specific effector proteins. By this means, activation of G-protein coupled receptors amplifies the effects of the environmental signal and initiates a cascade of intracellular events that ultimately leads to a defined cellular response. The family of G-protein coupled receptors function as a complex information processing network within the plasma membrane of the cell, acting to coordinate a cell's response to multiple environmental signals.

G-protein coupled receptors are characterized by the ability of agonists to promote the formation of a high affinity ternary complex between the agonist, the receptor and the G-protein (Figure 1). The α-subunit of the G protein contains a guanine nucleotide binding site which, in the high affinity ternary [G protein-receptor-agonist] complex, is occupied by GDP. In the presence of physiological concentrations of GTP, the GDP molecule in the guanine nucleotide binding site of the G protein is displaced by a GTP molecule. The binding of GTP dissociates the α subunit of the G protein from its $\beta\gamma$ subunits and from the receptor, thereby activating the G-protein to stimulate downstream effectors (adenylyl cyclase in the case of the β-adrenergic receptor (βAR)) and propagating the intracellular signal. Thus, the ternary complex is transient in the presence of physiological

GTP concentrations. Because the affinity of the agonist for the receptor-G protein complex is higher than its affinity for the uncomplexed receptor, one consequence of the destabilization of the ternary complex is a reduction in the affinity of the receptor for the agonist. Thus, the affinity of agonists for G-protein coupled receptors is a function of the efficiency with which the receptor is coupled to the G-protein. In contrast, antagonists bind with the same affinity to the receptor in the presence or absence of G-protein coupling.

The observation that agonist affinity can be reduced by conditions under which a receptor is not optimally coupled to its G-protein has important implications for the identification of agonists of G-protein coupled receptors, particularly identification based on ligand binding. If the receptor is not optimally coupled to the G-protein under the conditions of binding assays, an agonist will bind to the receptor with relatively low affinity. Thus, a screen that relies on a binding assay based on displacement of a radiolabeled ligand, although attractive for its ease and the potential for high throughput, poses the risk that a promising partial agonist might be overlooked because the agonist would bind predominantly to the low affinity state of the receptor, and thus would have low affinity in the binding assay. Consequently, functional assays are frequently used to screen for agonists of G-protein coupled receptors. However, functional assays (ranging from ex vivo muscle contraction assays to determination of second messenger levels in cells expressing exogenous cloned G-protein coupled receptors) are tedious and much more time-consuming than ligand binding assays, and hence are not readily adapted to high throughput screens. Because the modified receptors of the present invention bind agonists with high affinity in the presence or absence of the G-protein, they can be used in high throughput radioligand binding assays to screen for high affinity ligands, regardless of whether the ligands are agonists or antagonists.

G-protein coupled receptors consist of seven hydrophobic domains connecting eight hydrophilic domains. The hydrophobicity or hydrophilicity of the domains may be determined by standard hydropathy profiles, such as Kyte-Doolittle analysis (Kyte, J. and

Doolittle, R.J.F. *J. Mol. Biol.* 157: 105 (1982)). The receptors are thought to be oriented in the plasma membrane of the cell in such a way that the N-terminus of the receptor faces the extracellular space and the C-terminus of the receptor faces the cytoplasm, such that each of the hydrophobic domains crosses the plasma membrane. The receptors have been modeled and the putative boundaries of the extracellular, transmembrane and intracellular domains are generally agreed upon based on these models (for a review, see Baldwin, EMBO J. 12:1693, 1993). In general, the transmembrane domains are comprised of stretches of 20-25 amino acids in which most of the amino acid residues have hydrophobic side chains (including cysteine, methionine, phenylalanine, tyrosine, tryptophan, proline, glycine, alanine, valine, leucine, isoleucine), whereas the intracellular and extracellular loops are defined by contiguous stretches of several amino acids that have hydrophilic or polar side chains (including aspartate, glutamate, asparagine, glutamine, serine, threonine, histidine, lysine, and arginine). Polar amino acids, especially uncharged ones (such as serine, threonine, asparagine, and glutamine) are found in both transmembrane and extramembrane regions.

The extramembrane regions are characterized by contiguous stretches of three or more hydrophilic residues. In contrast, hydrophilic residues are found only in groups of 1-2, surrounded by hydrophobic residues, in the transmembrane domain. Thus, the transmembrane and extramembrane regions can be identified by the number of contiguous hydrophilic or hydrophobic amino acids in the primary sequence of the receptor, in addition to the constraints on the length of the hydrophobic segments given above. The boundaries between the transmembrane and extramembrane regions are often defined by the presence of charged or polar residues at the beginning or end of a stretch of hydrophobic amino acids. The locations of the mutations in the receptors of the present invention are described on the basis of these models and can be specifically defined by the specific amino acid numbers of the residues being mutated.

By these criteria, the third intracellular loop is defined as the hydrophilic loop connecting the hydrophobic, putative transmembrane domains V and VI. For example, in hamster β_2 adrenergic receptor, used to particularly exemplify the invention, the third intracellular loop would refer to amino acids 221 through 273 (Figure 2). In accordance with the principles described above, the beginning of this loop is defined by the presence of Arg221 (a charged residue at the end of the hydrophobic stretch of residues 198-220) and Lys273 (a charged residue at the beginning of the hydrophobic stretch of residues 274-298).

The present invention pertains to modified G-protein coupled receptors having deletions in the third intracellular domain. Methods of designing and making modified receptors are provided. The modified receptors are uncoupled from or are poorly coupled to their respective G-proteins. However, these modified receptors bind agonists with high affinity in the absence of G protein coupling. Because of their high intrinsic affinity for agonists, these modified receptors may be used in high throughput binding assays to identify compounds that bind to the receptor with high affinity, regardless of whether these compounds are agonists or antagonists. The invention includes the DNA encoding the modified receptors, the modified receptors, assays employing the modified receptors, cells expressing the modified receptors, and substances identified through the use of the modified receptors including specific modulators of the modified receptors. Modulators identified in this process are useful as therapeutic agents. Modulators, as described herein, include but are not limited to agonists, antagonists, suppressors and inducers.

SUMMARY OF THE INVENTION

Modified G-protein coupled receptors having deletions in the third intracellular domain are identified and methods of making the modified receptors are provided. The invention includes the modified receptors, assays employing the modified receptors, cells expressing the modified receptors, and compounds identified

through the use of the modified receptors, including modulators of the receptors. Modulators identified in this process are useful as therapeutic agents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic diagram of G-protein signal transduction system. The receptor is shown as a seven-helical bundle. α, ß, and γ indicate the three subunits of the G protein. E indicates an effector enzyme, such as adenylyl cyclase. The agonist (A) binding with high affinity to the receptor-G protein complex and with low affinity to the receptor alone is shown.

Figure 2. Schematic diagram of the hamster β_2 adrenergic receptor. The third intracellular loop comprises residues 221-273. The proximal and distal segments of this loop are drawn in cylinders.

Figure 3. Stimulation of cAMP production as a function of isoproterenol by the wild type β_3AR (closed circles) but not the modified D(227-234) (triangles) or D(277-289)β_3AR (squares).

Figure 4: Binding of an agonist and an antagonist to the wild type (open circles) and D(277-289) β_3AR (closed circles). Binding of the agonist isoproterenol (top panel) or the antagonist propranolol (bottom panel) was measured in competition with the radioligand ^{125}I-cyanopindolol.

Figure 5. Inhibition of adenylyl cyclase activity. A concentration dependent response curve of the ability of 5-HT to inhibit adenylate cyclase activity mediated by the wild type 5-HT1Dβ receptor is shown. However, in the histogram on the right of the figure, the inability of 100 mM 5-HT activating at the mutant receptor, D(231-239)5-HT1Dβ to produce an inhibition of adenylate cyclase activity is demonstrated. The results shown are from a typical experiment and were repeated three times and are representative of three independent mutant receptor cell lines [D(231-239)5-HT1Dβ clones 1, 21 and 65]. Formation of

^{32}P-cAMP from ^{32}P-ATP was measured in crude membrane preparations prepared from CHO cells stably expressing the appropriate receptors.

Figure 6. Table 1: Binding and functional parameters of the wild type and modified β_2AR.

Figure 7. Table 2: Binding parameters of the wild type and modified β_3AR.

Figure 8. Table 3: Radioligand binding properties of modified 5HT-1Dβ receptors. Presented in the table are the specific binding values (dpm) of 2 nm [^{3}H]5-HT observed in the presence and absence of the guanine nucleotide analog, GppNHp (100 mM). Also shown is the percentage inhibition of adenylate cyclase activity (%AC inhibition) for the respective cell lines. Results shown are from a typical experiment and were repeated three times.

DETAILED DESCRIPTION OF THE INVENTION

Modified G-protein coupled receptors having deletions in the third intracellular domain are identified and methods of making the modified receptors are provided. The modified receptors are uncoupled from or are poorly coupled to their respective G-proteins and may be used in assays to identify substances that bind to the receptor regardless of whether these substances are agonists or antagonists. The invention includes the modified receptors, assays employing the modified receptors, cells expressing the modified receptors, and compounds identified through the use of the modified receptors, including modulators of the receptors. Modulators identified in this process are useful as therapeutic agents. Modulators, as described herein, include but are not limited to agonists, antagonists, suppressors and inducers.

The term "G-protein coupled receptor" refers to any receptor protein that mediates its endogenous signal transduction

through activation of one or more guanine nucleotide binding regulatory proteins (G-proteins). These receptors share common structural features, including seven hydrophobic transmembrane domains. G-protein coupled receptors include receptors that bind to small biogenic amines, including but not limited to beta-adrenergic receptors (βAR), alpha-adrenergic receptors (αAR) and muscarinic receptors, as well as receptors whose endogenous ligands are peptides, such as neurokinin and glucagon receptors. Examples of βAR include beta-1, beta-2, and beta-3 adrenergic receptors. Examples of αAR include alpha-1a, alpha-1b, alpha-1c, alpha-2a, alpha-2b, and alpha-2c. Examples of muscarinic receptors include M1, M2, M3, M4 and M5. Examples of neurokinin receptors include NK1, NK2 and NK3. Other examples of G-protein coupled receptors include but are not limited to adenosine 2 receptor, alpha-2 adrenergic receptors, type-1 angiotensin II receptor, cholecytokinin B receptor, gastrin receptor, somatostatin receptor, 5-hydroxytryptamine 1 beta receptor, A2 adenosine receptor, Burkitt's lymphoma receptor, neuropeptide Y receptor, tachykinin receptor, serotonin receptor, formyl peptide receptor like-1, tyramine receptor, muscarinic acetylcholine receptor, certain endothelin receptors, complement protein 5a receptor, choriogonadotropic hormone receptor, high affinity interleukin 8 receptor, follicle stimulating hormone receptor, dopamine D1 receptor, C5a anaphylotoxin receptor, histamine H2 receptor, substance P receptor, thyrotropin receptor and, luteininzing hormone receptor. G-protein coupled receptors have been isolated from a variety of animals, including but not limited to humans, cows, goats, mice, pigs and rats.

Modified eceptors may include genetic variants, both natural and induced. Induced modified receptors may be derived by a variety of methods, including but not limited to, site-directed mutagenesis. Techniques for nucleic acid and protein manipulation are well-known in the art and are described generally in Methods in Enzymology and in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory (1989).

It is known that there is a substantial amount of redundancy in the various codons which code for specific amino acids. Therefore, this invention is also directed to those DNA sequences which contain alternative codons which code for the eventual translation of the identical amino acid. For purposes of this specification, a sequence bearing one or more replaced codons will be defined as a degenerate variation. Also included within the scope of this invention are mutations either in the DNA sequence or the translated protein which do not substantially alter the ultimate physical properties of the expressed protein. For example, substitution of valine for leucine, arginine for lysine, or asparagine for glutamine may not cause a change in functionality of the polypeptide.

It is known that DNA sequences coding for a peptide may be altered so as to code for a peptide having properties that are different than those of the naturally-occurring peptide. Methods of altering the DNA sequences include, but are not limited to site directed mutagenesis. Examples of altered properties include but are not limited to changes in the affinity of an enzyme for a substrate or a receptor for a ligand.

As used herein, a "functional derivative" of a modified receptor is a compound that possesses a biological activity (either functional or structural) that is substantially similar to the biological activity of the modified receptor. The term "functional derivative" is intended to include the "fragments," "variants," "degenerate variants," "analogs" and "homologues" or to "chemical derivatives" of modified receptors. The term "fragment" is meant to refer to any polypeptide subset of modified receptors. The term "variant" is meant to refer to a molecule substantially similar in structure and function to either the entire modified receptor molecule or to a fragment thereof. A molecule is "substantially similar" to a modified receptor if both molecules have substantially similar structures or if both molecules possess similar biological activity. Therefore, if the two molecules possess substantially similar activity,

they are considered to be variants even if the structure of one of the molecules is not found in the other or even if the two amino acid sequences are not identical.

The term "analog" refers to a molecule substantially similar in function to either the entire modified receptor molecule or to a fragment thereof.

"Substantial homology" or "substantial similarity", when referring to nucleic acids means that the segments or their complementary strands, when optimally aligned and compared, are identical with appropriate nucleotide insertions or deletions, in at least 75% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize to a strand or its complement.

The nucleic acids claimed herein may be present in whole cells or in cell lysates or in a partially purified or substantially purified form. A nucleic acid is considered substantially purified when it is purified away from environmental contaminants. Thus, a nucleic acid sequence isolated from cells is considered to be substantially purified when purified from cellular components by standard methods while a chemically synthesized nucleic acid sequence is considered to be substantially purified when purified from its chemical precursors.

Nucleic acid compositions of this invention may be derived from genomic DNA or cDNA, prepared by synthesis or by a combination of techniques.

The natural or synthetic nucleic acids encoding the modified G-coupled protein receptors of the present invention may be incorporated into expression vectors. Usually the expression vectors incorporating the modified receptors will be suitable for replication in a host. Examples of acceptable hosts include, but are not limited to, prokaryotic and eukaryotic cells.

The phrase "recombinant expression system" as used herein means a substantially homogenous culture of suitable host organisms that stably carry a recombinant expression vector. Examples of suitable hosts include, but are not limited to, bacteria, yeast, fungi, insect cells, plant cells and mammalian cells. Generally, cells of the

expression system are the progeny of a single ancestral transformed cell.

The cloned modified receptor DNA obtained through the methods described herein may be recombinantly expressed by molecular cloning into an expression vector containing a suitable promoter and other appropriate transcription regulatory elements, and transferred into prokaryotic or eukaryotic host cells to produce recombinant modified receptor. Techniques for such manipulations are fully described in Sambrook, J., et al., supra, and are well known in the art.

Expression vectors are defined herein as DNA sequences that are required for the transcription of cloned copies of genes and the translation of their mRNAs in an appropriate host. Such vectors can be used to express eukaryotic genes in a variety of hosts such as bacteria, bluegreen algae, plant cells, insect cells, fungal cells and animal cells.

Specifically designed vectors allow the shuttling of DNA between hosts such as bacteria-yeast or bacteria-animal cells or bacteria-fungal cells or bacteria-invertebrate cells. An appropriately constructed expression vector should contain: an origin of replication for autonomous replication in host cells, selectable markers, a limited number of useful restriction enzyme sites, a potential for high copy number, and active promoters. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and initiate RNA synthesis. A strong promoter is one which causes mRNAs to be initiated at high frequency. Expression vectors may include, but are not limited to, cloning vectors, modified cloning vectors, specifically designed plasmids or viruses.

A variety of mammalian expression vectors may be used to express recombinant modified receptor in mammalian cells. Commercially available mammalian expression vectors which may be suitable for recombinant modified receptor expression, include but are not limited to, pcDNA3 (Invitrogen), pMC1neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2-neo (ATCC

37593) pBPV-1(8-2) (ATCC 37110), pdBPV-MMTneo(342-12) (ATCC 37224), pRSVgpt (ATCC 37199), pRSVneo (ATCC 37198), pSV2-dhfr (ATCC 37146), pUCTag (ATCC 37460), and λZD35 (ATCC 37565).

A variety of bacterial expression vectors may be used to express recombinant modified receptor in bacterial cells. Commercially available bacterial expression vectors which may be suitable for recombinant modified receptor expression include, but are not limited to pET11a (Novagen), lambda gt11 (Invitrogen), pcDNAII (Invitrogen), pKK223-3 (Pharmacia).

A variety of fungal cell expression vectors may be used to express recombinant modified receptor in fungal cells. Commercially available fungal cell expression vectors which may be suitable for recombinant modified receptor expression include but are not limited to pYES2 (Invitrogen), *Pichia* expression vector (Invitrogen).

A variety of insect cell expression vectors may be used to express recombinant receptor in insect cells. Commercially available insect cell expression vectors which may be suitable for recombinant expression of modified receptor include but are not limited to pBlue Bac III (Invitrogen).

An expression vector containing DNA encoding modified receptor may be used for expression of modified receptor in a recombinant host cell. Recombinant host cells may be prokaryotic or eukaryotic, including but not limited to bacteria such as E. coli, fungal cells such as yeast, mammalian cells including but not limited to cell lines of human, bovine, porcine, monkey and rodent origin, and insect cells including but not limited to Drosophila and silkworm derived cell lines. Cell lines derived from mammalian species which may be suitable and which are commercially available, include but are not limited to, L cells L-M(TK$^-$) (ATCC CCL 1.3), L cells L-M (ATCC CCL 1.2), 293 (ATCC CRL 1573), Raji (ATCC CCL 86), CV-1 (ATCC CCL 70), COS-1 (ATCC CRL 1650), COS-7 (ATCC CRL 1651), CHO-K1 (ATCC CCL 61), 3T3 (ATCC CCL

92), NIH/3T3 (ATCC CRL 1658), HeLa (ATCC CCL 2), C127I (ATCC CRL 1616), BS-C-1 (ATCC CCL 26) and MRC-5 (ATCC CCL 171).

The expression vector may be introduced into host cells via any one of a number of techniques including but not limited to transformation, transfection, lipofection, protoplast fusion, and electroporation. The expression vector-containing cells are clonally propagated and individually analyzed to determine whether they produce modified receptor protein. Identification of modified receptor expressing host cell clones may be done by several means, including but not limited to immunological reactivity with anti-modified receptor antibodies.

Expression of modified receptor DNA may also be performed using *in vitro* produced synthetic mRNA or native mRNA. Synthetic mRNA or mRNA isolated from modified receptor producing cells can be efficiently translated in various cell-free systems, including but not limited to wheat germ extracts and reticulocyte extracts, as well as efficiently translated in cell based systems, including but not limited to microinjection into frog oocytes, with microinjection into frog oocytes being preferred.

The term "substantial homology", when referring to polypeptides, indicates that the polypeptide or protein in question exhibits at least about 30% homology with the naturally occurring protein in question, usually at least about 65% homology.

The modified receptors may be expressed in an appropriate host cell and used to discover compounds that affect the modified receptor. Preferably, the modified receptors are expressed in a mammalian cell line, including but not limited to, COS-7, CHO or L cells, or an insect cell line, including but not limited to Sf9 and Sf21, and may be used to discover ligands that bind to the receptor and alter or stimulate its function. The modified receptors may also be produced in bacterial, fungal or yeast expression systems.

The expression of the modified receptor may be detected by use of a radiolabeled ligand specific for the receptor. For the β_2

adrenergic receptor used herein to exemplify the invention, such a ligand may be ^{125}I-iodocyanopindolol (^{125}I-CYP).

The specificity of binding of compounds showing affinity for the modified receptors is shown by measuring the affinity of the compounds for cells transfected with the cloned modified receptor or for membranes from these cells. Expression of the cloned modified receptor and screening for compounds that inhibit the binding of radiolabeled ligand to these cells provides a rational way for selection of compounds with high affinity for the receptor. These compounds may be agonists or antagonists of the receptor. Because the modified receptor does not couple well to G proteins, the agonist activity of these compounds is best assessed by using the wild-type receptor, either natively expressed in tissues or cloned and exogenously expressed.

Once the modified receptor is cloned and expressed in a mammalian cell line, such as COS-7 cells or CHO cells, the recombinant modified receptor is in a well-characterized environment. The membranes from the recombinant cells expressing the modified receptor are then isolated according to methods known in the art. The isolated membranes may be used in a variety of membrane-based receptor binding assays. Because the modified receptor has a high affinity for agonists, ligands (either agonists or antagonists) may be identified by standard radioligand binding assays. These assays will measure the intrinsic affinity of the ligand for the receptor.

The present invention provides methods of generating modified G-protein coupled receptors. Such methods generally comprise the deletion of at least one nucleotide from the third intracellular domain of the receptor. Additional methods include, but are not limited to, enzymatic or chemical removal of amino acids from the third intracellular domain of the receptor. One method of generating modified G-protein receptors comprises:

(a) isolating DNA encoding a G-protein coupled receptor;

(b) altering the DNA of step (a) by deleting at least one nucleotide from DNA encoding the third intracellular domain of the G-protein coupled receptor;

(c) isolating the altered DNA;

(d) expressing the altered DNA; and

(e) recovering the modified G-protein coupled receptor.

The third intracellular domain of a G-protein coupled receptor is located between the fifth and sixth hydrophobic transmembrane domains of the receptor (Figure 2).

The present invention provides methods of identifying compounds that bind to modified G-protein coupled receptors. Methods of identifying compounds are exemplified by an assay, comprising:

a) cloning the G-protein coupled receptor;

b) altering the DNA sequence encoding the third intracellular domain of the cloned G-protein coupled receptor;

c) splicing the altered receptor into an expression vector to produce a construct such that the altered receptor is operably linked to transcription and translation signals sufficient to induce expression of the receptor upon introduction of the construct into a prokaryotic or eukaryotic cell;

d) introducing the construct into a prokaryotic or eukaryotic cell which does not express the altered receptor in the absence of the introduced construct; and

e) incubating cells or membranes isolated from cells produced in step c with a quantifiable compound known to bind to the receptors, and subsequently adding test compounds at a range of concentrations so as to compete the quantifiable compound from the receptor, such that an IC_{50} for the test compound is obtained as the concentration of test compound at which 50% of the quantifiable compound becomes displaced from the receptor.

The present invention is also directed to methods for screening for compounds which modulate the expression of DNA or RNA encoding modified receptors or which modulate the function of modified receptor protein. Compounds which modulate these activities may be DNA, RNA, peptides, proteins, or non-proteinaceous organic molecules. Compounds may modulate by increasing or attenuating the expression of DNA or RNA encoding

modified receptor, or the function of modified receptor protein. Compounds that modulate the expression of DNA or RNA encoding modified receptor or the function of modified receptor protein may be detected by a variety of assays. The assay may be a simple "yes/no" assay to determine whether there is a change in expression or function. The assay may be made quantitative by comparing the expression or function of a test sample with the levels of expression or function in a standard sample.

Kits containing modified receptor DNA, antibodies to modified receptor, or modified receptor protein may be prepared. Such kits are used to detect DNA which hybridizes to modified receptor DNA or to detect the presence of modified receptor protein or peptide fragments in a sample. Such characterization is useful for a variety of purposes including but not limited to forensic, taxonomic or epidemiological studies.

The DNA molecules, RNA molecules, recombinant protein and antibodies of the present invention may be used to screen and measure levels of modified receptor DNA, modified receptor RNA or modified receptor protein. The recombinant proteins, DNA molecules, RNA molecules and antibodies lend themselves to the formulation of kits suitable for the detection and typing of modified receptor. Such a kit would comprise a compartmentalized carrier suitable to hold in close confinement at least one container. The carrier would further comprise reagents such as recombinant modified receptor protein or anti-modified receptor antibodies suitable for detecting modified receptor. The carrier may also contain a means for detection such as labeled antigen or enzyme substrates or the like.

Pharmaceutically useful compositions comprising modulators of modified receptor activity, may be formulated according to known methods such as by the admixture of a pharmaceutically acceptable carrier. Examples of such carriers and methods of formulation may be found in Remington's Pharmaceutical Sciences. To form a pharmaceutically acceptable

composition suitable for effective administration, such compositions will contain an effective amount of the protein, DNA, RNA, or modulator.

Therapeutic or diagnostic compositions of the invention are administered to an individual in amounts sufficient to treat or diagnose disorders. The effective amount may vary according to a variety of factors such as the individual's condition, weight, sex and age. Other factors include the mode of administration.

The pharmaceutical compositions may be provided to the individual by a variety of routes such as subcutaneous, topical, oral and intramuscular.

The term "chemical derivative" describes a molecule that contains additional chemical moieties which are not normally a part of the base molecule. Such moieties may improve the solubility, half-life, absorption, etc. of the base molecule. Alternatively the moieties may attenuate undesirable side effects of the base molecule or decrease the toxicity of the base molecule. Examples of such moieties are described in a variety of texts, such as Remington's Pharmaceutical Sciences.

Compounds identified according to the methods disclosed herein may be used alone at appropriate dosages. Alternatively, co-administration or sequential administration of other agents may be desirable.

The present invention also has the objective of providing suitable topical, oral, systemic and parenteral pharmaceutical formulations for use in the novel methods of treatment of the present invention. The compositions containing compounds identified according to this invention as the active ingredient can be administered in a wide variety of therapeutic dosage forms in conventional vehicles for administration. For example, the compounds can be administered in such oral dosage forms as tablets, capsules (each including timed release and sustained release formulations), pills, powders, granules, elixirs, tinctures, solutions, suspensions, syrups and emulsions, or by injection. Likewise, they

may also be administered in intravenous (both bolus and infusion), intraperitoneal, subcutaneous, topical with or without occlusion, or intramuscular form, all using forms well known to those of ordinary skill in the pharmaceutical arts.

Advantageously, compounds of the present invention may be administered in a single daily dose, or the total daily dosage may be administered in divided doses of two, three or four times daily. Furthermore, compounds for the present invention can be administered in intranasal form via topical use of suitable intranasal vehicles, or via transdermal routes, using those forms of transdermal skin patches well known to those of ordinary skill in that art. To be administered in the form of a transdermal delivery system, the dosage administration will, of course, be continuous rather than intermittent throughout the dosage regimen.

For combination treatment with more than one active agent, where the active agents are in separate dosage formulations, the active agents can be administered concurrently, or they each can be administered at separately staggered times.

The dosage regimen utilizing the compounds of the present invention is selected in accordance with a variety of factors including type, species, age, weight, sex and medical condition of the patient; the severity of the condition to be treated; the route of administration; the renal and hepatic function of the patient; and the particular compound thereof employed. A physician or veterinarian of ordinary skill can readily determine and prescribe the effective amount of the drug required to prevent, counter or arrest the progress of the condition. Optimal precision in achieving concentrations of drug within the range that yields efficacy without toxicity requires a regimen based on the kinetics of the drug's availability to target sites. This involves a consideration of the distribution, equilibrium, and elimination of a drug.

The modified G-protein coupled receptors of the present invention are exemplified herein by the hamster beta-2 ($\beta 2$) adrenergic receptor, the human $\beta 3$ receptor and the human 5HT-1Dβ receptor.

Deletion mutagenesis of the β2-adrenergic receptor has shown that none of the hydrophobic clusters of amino acids (the putative transmembrane helices) could be deleted without substantial loss of binding. In contrast, most of the connecting loops could be deleted without affecting the ligand binding properties of the receptor. This indicates that these hydrophilic loops are not required for ligand binding to the receptor, suggesting that the ligand binding pocket is located predominantly within the transmembrane domain of the protein (Strader, et al *FASEB J* .3: 182-183 (1989)). Deletions in the connecting loops that were large enough to encompass the entire loop led to steric problems, resulting in incorrect processing of the protein (Dixon, et al. *EMBO J.* 6: 3269-3275 (1987)). Certain connecting loop deletion mutations, however, led to loss of functional activation of adenylyl cyclase by the receptor. For example, deletion of the carboxy terminal region of the third intracellular loop attenuated the ability of the receptor to activate adenylyl cyclase, and deletion of the amino terminal portion of this loop abolished adenylyl cyclase activation (Strader, et al *J. Biol. Chem.* 262: 16439-16443 (1987)). Moreover, the agonist binding isotherms for these modified receptors displayed a single affinity site, suggesting altered G protein interactions. Since these modified receptors also retain their functional activation of Na^+-H^+ exchange, which is mediated through a different G protein (Barber, et al. *Mol. Pharm.* 41: 1056-1060 (1992)), the deletions appear not to result in gross structural perturbations of the receptor, suggesting that the changes seen in adenylyl cyclase activation are due to alteration of a specific G protein interaction. Subsequent amino acid replacements in the third intracellular loop confirmed the role of this region in G protein interaction (Cheung, et al. *Mol. Pharm.* 41: 1061-1065 (1992)).

The following examples are provided to further define the invention without, however, limiting the invention to the particulars of the examples.

EXAMPLE 1

Deletion of 6-12 amino acids at the N-terminal portion of the third intracellular loop of the hamster β2 adrenergic receptor

Modified receptor D(222-229)β2AR was described in Strader et al. (*J. Biol. Chem.* 262:16349, 1987). A modified cDNA encoding the hamster β2AR in which residues 222-229 (Val-Phe-Gln-Val-Ala-Lys-Arg-Gln) are deleted was constructed by standard oligonucleotide-directed mutagenesis procedures.

The modified receptor is designed so as to disrupt the proximal portion of the third intracellular loop, without affecting the adjacent fifth transmembrane helix. Thus, the charged amino acid (Arg221) that delineates the bottom of helix 5 is left intact in the D(222-229) modified receptor, while the following eight amino acids are deleted. The size of the deletion in the present invention may vary from six to 13 amino acids in these regions, beginning immediately after the charged residue at the end of transmembrane helix 5.

EXAMPLE 2

Deletion of amino acids at the C-terminal portion of the third intracellular loop of the hamster β2 adrenergic receptor

Modified receptor D(258-270)β2AR was described in Strader et al. (*J. Biol. Chem.* 262:16349, 1987). A modified cDNA encoding the hamster β2AR in which residues 258-270 (Leu-Arg-Arg-Ser-Ser-Lys-Phe-Cys-Leu-Lys-Glu-His-Lys) were deleted was constructed by standard oligonucleotide-directed mutagenesis procedures.

The modified receptor is designed so as to disrupt the distal portion of the third intracellular loop, without affecting the adjacent sixth transmembrane helix. Thus, the charged amino acid (Lys273) that delineates the bottom of helix 6 is left intact in the D(258-270) modified receptor, while the nearby proximal residues 258-270 are deleted. The size of the deletion in the present invention may vary from six to 13 amino acids in these regions, ending 1-3 residues before the charged residue at the beginning of helix 6.

EXAMPLE 3

Expression and characterization of the altered β2 adrenergic receptor.

COS-7 cells are transfected with the modified receptor cDNA subcloned into a eukaryotic expression vector such as the eukaryotic expression vector pcDNA I/neo (Invitrogen). Cells are harvested after incubation for about 60-72 h. Membranes containing the expressed receptor protein are prepared as described (C. D. Strader et al., *Proc. Natl. Acad. Sci. U.S.A.* 84, 4384-4388 (1987).

Binding reactions are performed in a final volume of 250 µl of TME buffer (75 mM Tris; 12.5 mM $MgCl_2$; 1.5 mM EDTA, pH 7.5) as described (Strader, et al *J. Biol. Chem.* 262: 16439 (1987)). Adenylyl cyclase activity is measured as described (Strader, et al *J. Biol. Chem.* 262: 16439 (1987)), with cAMP determined by the method of Salomon (*Anal. Biochem.* 58: 541-548 (1974)).

Membranes prepared from the COS-7 cells transfected with a vector containing either the wild type or the modified receptor cDNA specifically bind the β receptor antagonist ^{125}I-CYP. However, the modified receptor is characterized by an absence of coupling to Gs, an inability to mediate the activation of adenylyl cyclase, and an increased affinity for agonists.

As shown in Table 1, the modified D(222-229)β_2AR, when expressed in L cells, does not stimulate adenylyl cyclase activation in response to the agonist isoproterenol. In contrast, when the wild type receptor is expressed in the same cell line, adenylyl cyclase activity is stimulated by 3.2 fold, with an EC_{50} of 15 nM. The modified receptor retains its ability to stimulate Na^+-H^+ exchange, indicating that some level of coupling to a G-protein other than G_S is retained (Barber et al. Mol. Pharm. 41, 1056, 1992). Similarly, D(258-270)βAR shows impaired cAMP stimulation compared to the wild type receptor, with only a small (1.3 fold) stimulation over basal levels.

These modified receptors have increased affinity for agonists when compared to the wild type receptor. This is shown in Table 1, where the modified D(222-229) receptor binds the agonist

isoproterenol with a single high affinity of 6 nM. The high affinity of the agonist for the modified receptor is not affected by agents that uncouple the receptor from the G protein; such agents include the nonhydrolyzable GTP analog GppNHp, sodium fluoride, and the detergent digitonin. In contrast, the wild type receptor binds isoproterenol with two affinity states: a high affinity state (K_d = 3 nM) indicative of binding to the receptor-G protein complex, and a low affinity state (K_d = 200 nM) reflecting binding to the uncoupled receptor alone (Table 1). In the presence of agents that interfere with G protein coupling (GppNHp is such an agent shown in Table 1), the agonist binds to the wild type receptor with a single low affinity state (K_d = 200 nM).

The data in Table 1 demonstrate that when the receptor is not optimally coupled to the G protein, a binding assay using the modified receptor will detect agonists with more sensitivity than will the identical binding assay using the wild type receptor. Similarly, D(258-270)βAR binds to the agonist isoproterenol with a single high affinity of 8 nM, which is not significantly affected by the addition of Gpp(NH)p.

EXAMPLE 4

Screening Assay using D(222-229) βAR or D(258-270)βAR

Transfected cells expressing recombinant modified receptor may be used to identify compounds that bind to the receptor with high affinity. This may be accomplished in a variety of ways, such as by incubating the test compound in a final volume of 0.25 ml of TME buffer with membranes containing 5-7 pM of the modified β_2AR and 35 pM ^{125}I-CYP for 1 hour at 25°. The reaction is stopped by filtration over GF/C glass fiber filters, washing with 3 x 5 ml of cold TME buffer, and counting the filters in a gamma counter to measure bound radioactivity. This assay will detect a compound that has a high intrinsic affinity for the receptor. Such compounds may be either agonists or antagonists.

EXAMPLE 5

Construction of Modified D(227-234) Beta-3 Adrenergic Receptor

Modified receptor D(227-234) β3AR was constructed by digesting the wild-type human β3 receptor cDNA (Granneman, et al. *Mol. Pharm.* 42: 964-970 (1992))) with AccI and PvuII, followed by re-ligation with a linker adaptor. The sequence of the linker adaptor is:

5'CTACGCGCGG3'/3'TGCGCGCC5' (SEQ ID NO:1).

The modified DNA sequence encodes a β3AR lacking 8 amino acid residues (VFVVATRQ) at the N-terminal portion of the third intracellular loop. The nucleotide sequence of the modified receptor was confirmed by DNA sequencing. As was the case for the modified β2 receptors, this modified β3 receptor is designed so as to disrupt the proximal portion of the third intracellular loop, without affecting the adjacent fifth transmembrane helix. Thus, the charged amino acid (Arg226) that delineates the bottom of helix 5 is left intact in the D(227-234) modified receptor, while the eight amino acids which follow it are deleted. The size of the deletion in the present invention may vary from six to 13 amino acids in this region, beginning immediately after the charged residue at the bottom of transmembrane helix 5.

EXAMPLE 6

Construction of Modified D(277-289) Beta-3 Adrenergic Receptor

Modified D(277-289), lacking 13 residues at the C-terminal portion of the third intracellular loop, was prepared by standard PCR-based mutagenesis procedures. The nucleotide sequences of the modified receptors were confirmed by DNA sequencing. As was the case for the modified β2 receptors, this modified β3 receptor is designed so as to disrupt the distal portion of the third intracellular loop, without affecting the adjacent sixth transmembrane helix. Thus, the polar amino acids (C292,T293) that define the bottom of helix 6 are left intact, while the nearby proximal residues 277-289 are deleted. The

size of the deletion in the present invention may vary from six to 13 amino acids in this region, ending immediately before the polar residues at the bottom of helix 6.

EXAMPLE 7

Expression and characterization of the modified β3AR

The modified receptor was subcloned into the expression vector pRC/CMV (Invitrogen, San Diego, CA) and expressed in mouse L cells by DEAE-Dextran transfection. 72 hours after transfection, cells were harvested for radioligand binding or adenylyl cyclase assays.

For binding assays, the membranes were prepared by harvesting the cells in ice-cold lysis buffer (5 mg Tris, pH 7.4; 2 mM EDTA), followed by 15 min centrifugation at 38,000 x g. The membrane pellet was then resuspended in TME buffer. Equilibrium binding to the wild type or modified β3AR was performed in a final volume of 0.25 ml containing membranes, 240 pM ^{125}I-CYP, and serial dilution of the competing ligands. Binding reactions were incubated for 90 min at 23°C, and terminated by rapid filtration over GF/C filters pre-soaked in 0.1% polyethyeneamine. The radioactivity was quantified with a Packard gamma counter.

For adenylyl cyclase activity, cells are harvested in PBS with 5 mM EDTA, pelleted and, then resuspended in ACC buffer (75 mM Tris, pH 7.4; 250 mM sucrose; 12.5 mM $MgCl_2$; 1.5 mM EDTA; 1 µM ascorbic acid; 0.6 mM 3-isobutyl-1-1 - methylxanthine). The cells are incubated with various concentrations of test compound (usually agonist compound) for 45 min at room temperature, and the reaction terminated by boiling for 3 min. The concentration of cAMP in the lysate was determined via protein kinase A (PKA) binding assay (Barton, A.C., Black, L.E., Sibley, D.R., *Mol. Pharmacy.* 39:650-658, 1991) or an automated cAMP IRA assay (At Instruments, MD). For the PKA binding assay, the lysate was incubated with 3.6 nM ^{3}H-cAMP and 5 µg of PKA in a final volume of 185 µl for 2 to 24 hours at 4° C, followed by rapid filtration over GF/C filters with cold washing buffer (20 mM potassium phosphate, pH 6.0). The radioactivity on the filter

was then quantified on a beta counter. The final concentration of cAMP was determined according to the standard curve of cAMP. The data for both binding and cyclase assays were analyzed by using graphed software (San Diego, CA).

Figure 3 shows that, when stimulated with the beta agonist isoproterenol, there is a four-fold increase in the production of cAMP in L cells transfected with the wild type human β3AR, with a EC_{50} of $2.7 \pm 0.5 \times 10^{-8}$ M (n=4). By contrast, the β3AR-mediated production of cAMP is essentially abolished in cells transfected with modified receptor D(227-234)β3AR and strongly attenuated in cells expressing the D(277-289) modified receptor.

Radioligand binding with ^{125}I-CYP indicates that the wild type β3AR displays two affinity sites for isoproterenol binding: a high affinity site (28%, $IC_{50}=5 \times 10^{-8}$ M), and a low affinity site (72%, $IC_{50}=2.6 \times 10^{-6}$ M). Deletion of residues 227-234 or residues 277-289 from the β3AR results in a single high affinity binding state (Table 2 and Figure 4). No increase in binding affinity is observed for the βAR antagonist propranolol for either modified receptor (Figure 4).

These modified β3 receptors can therefore be used in a screening assay to detect compounds that bind with high affinity to the β3 adrenergic receptor, regardless of whether these compounds are agonists or antagonists.

EXAMPLE 8

Construction of Modified D(231-238)5HT-1Dβ Receptor

Modified receptor D(231-238)5HT-1Dβ receptor was constructed from the wild-type human 5HT-1Dβ receptor cDNA (Jin, et al J. Biol. Chem. 267: 5735 (1992)) by standard mutagenesis techniques. The modified 5HT-1Dβ receptor lacks 8 amino acid residues (IYVEARSR) at the N-terminal portion of the third intracellular loop. The nucleotide sequences of the modified receptors were confirmed by DNA sequencing. As was the case for the modified β2 and β3 receptors, this modified 5HT-1Dβ receptor is designed so as to disrupt the proximal portion of the third intracellular loop without affecting the

adjacent fifth transmembrane helix. Thus, the charged amino acid (Arg230) that delineates the bottom of helix 5 is left intact in the modified receptor, while the following eight amino acids are deleted. The size of the deletion in the present invention may vary from six to 13 amino acids in this region, beginning immediately after the charged residue at the end of transmembrane helix 5.

EXAMPLE 9

Expression and Characterization of Modified D(231-238)5HT-1Dβ Receptor

The modified receptor was subcloned into a mammalian expression vector and expressed in CHO cells using standard transfection methods. Stable cell lines were selected by G-418 resistance and used for radioligand binding or adenylyl cyclase assays.

For binding assays, the membranes were prepared by harvesting the cells in ice-cold lysis buffer (5 mg Tris, pH 7.4; 2 mM EDTA), followed by 15 min centrifugation at 38,000 x g. The membrane pellet was then resuspended in buffer A. Equilibrium binding to the wild type or modified 5HT-1Dβ was performed in a mixture containing membranes, 5 nM ^{3}H 5-hydroxytryptamine, and serial dilutions of the competing ligands. Binding reactions were incubated for x min at 23°C, and terminated by rapid filtration over GF/C filters. The bound radioactivity was quantified with a gamma counter.

Adenylyl cyclase activity was measured essentially as described by McAllister et al. (McAllister, G., Charlesworth, A., Snodin, C., Beer, M. S., Noble, A. J., Middlemiss, D. N., Iversen, L. L., and Whiting, P., 1992, PNAS 89:5517-5521), with the addition of forskolin. Inhibition of the forskolin-stimulated response by receptor agonists, including 5-hydroxytryptamine (serotonin), was determined.

Figure 5 shows that, when stimulated with the agonist serotonin, there is a 50% inhibition in the forskolin-stimulated production of cAMP in cells expressing with the wild type human 5HT-1Dβ receptor, with a EC_{50} of 30 nM. By contrast, the agonist-

mediated inhibition of cAMP production is essentially abolished in cells transfected with modified receptor D(231-239)5HT-1Dβ.

Radioligand binding studies at the wild type 5-HT1Dβ receptor indicate that when the guanine nucleotide analogue, GppNHp (guanylylimidodiphosphate) is present (100 mM), agonist binding (2 nM ^{3}H-5-HT) is reduced by approximately 50-60% (Table 3). This is thought to be a result of the guanine nucleotide converting the receptor to the low affinity state. However, in three independent clones expressing the modified receptor, D(231-239)5-HT1Dβ (clones 1, 21 and 65), no significant inhibition of agonist binding is observed, suggesting that the modified receptor is permanently in the high affinity state.

This modified 5HT-1Dβ receptor can therefore be used in a screening assay to detect compounds that bind with high affinity to the 5HT-1Dβ receptor, regardless of whether these compounds are agonists or antagonists.

EXAMPLE 10

Cloning and Expression of Modified Receptor cDNA into Bacterial Expression Vectors

Recombinant modified receptor is produced in a bacterial expression system such as E. coli. The modified receptor expression cassette is transferred into an E. coli expression vector; expression vectors include but are not limited to, the pET series (Novagen). The pET vectors place modified receptor expression under control of the tightly regulated bacteriophage T7 promoter. Following transfer of this construct into an E. coli host which contains a chromosomal copy of the T7 RNA polymerase gene driven by the inducible lac promoter, expression of modified receptor is induced by addition of an appropriate lac substrate (IPTG) is added to the culture. The levels of expressed modified receptor are determined by the assays described herein.

EXAMPLE 11

Cloning and Expression of Modified Receptor cDNA into a Vector for Expression in Insect Cells

Baculovirus vectors derived from the genome of the AcNPV virus are designed to provide high level expression of cDNA in the Sf9 line of insect cells (ATCC CRL# 1711). Recombinant baculovirus expressing modified receptor cDNA is produced by the following standard methods (InVitrogen Maxbac Manual): the modified receptor cDNA constructs are ligated into the polyhedrin gene in a variety of baculovirus transfer vectors, including the pAC360 and the BlueBac vector (InVitrogen). Recombinant baculoviruses are generated by homologous recombination following co-transfection of the baculovirus transfer vector and linearized AcNPV genomic DNA [Kitts, P.A., *Nuc. Acid. Res.* 18, 5667 (1990)] into Sf9 cells. Recombinant pAC360 viruses are identified by the absence of inclusion bodies in infected cells and recombinant pBlueBac viruses are identified on the basis of ß-galactosidase expression (Summers, M. D. and Smith, G. E., Texas Agriculture Exp. Station Bulletin No. 1555). Following plaque purification, modified receptor expression is measured.

Authentic modified receptor is found in association with the infected cells. Active modified receptor is extracted from infected cells by hypotonic or detergent lysis.

Alternatively, the modified receptor is expressed in the *Drosophila* Schneider 2 cell line by cotransfection of the Schneider 2 cells with a vector containing the modified receptor DNA downstream and under control of an inducible metallothionin promoter, and a vector encoding the G418 resistant neomycin gene. Following growth in the presence of G418, resistant cells are obtained and induced to express modified receptor by the addition of $CuSO_4$. Identification of modulators of the modified receptor is accomplished by assays using either whole cells or membrane preparations.

EXAMPLE 12

Cloning of Modified Receptor cDNA into a yeast expression vector

Recombinant modified receptor is produced in the yeast S. cerevisiae following the insertion of the modified receptor cDNA cistron into expression vectors designed to direct the intracellular or extracellular expression of heterologous proteins. In the case of intracellular expression, vectors such as EmBLyex4 or the like are ligated to the modified receptor cistron [Rinas, U. et al., *Biotechnology* 8, 543-545 (1990); Horowitz B. et al., *J. Biol. Chem.* 265, 4189-4192 (1989)]. For extracellular expression, the modified receptor cistron is ligated into yeast expression vectors which fuse a secretion signal. The levels of expressed modified receptor are determined by the assays described herein.

EXAMPLE 13

Purification of Recombinant Modified Receptor

Recombinantly produced modified receptor may be purified by a variety of procedures, including but not limited to antibody affinity chromatography.

Modified receptor antibody affinity columns are made by adding the anti-modified receptor antibodies to Affigel-10 (Biorad), a gel support which is pre-activated with N-hydroxysuccinimide esters such that the antibodies form covalent linkages with the agarose gel bead support. The antibodies are then coupled to the gel via amide bonds with the spacer arm. The remaining activated esters are then quenched with 1 M ethanolamine HCl (pH 8). The column is washed with water followed by 0.23 M glycine HCl (pH 2.6) to remove any non-conjugated antibody or extraneous protein. The column is then equilibrated in phosphate buffered saline (pH 7.3) together with appropriate membrane solubilizing agents such as detergents, and the cell culture supernatants or cell extracts containing solubilized modified receptor or modified receptor subunits are slowly passed through the column. The column is then washed with phosphate-buffered saline (PBS)

supplemented with detergents until the optical density (A_{280}) falls to background; then the protein is eluted with 0.23 M glycine-HCl (pH 2.6) supplemented with detergents. The purified modified receptor protein is then dialyzed against PBS.

EXAMPLE 14

Cloning and Expression of Modified Receptor in Mammalian Cell System

A modified receptor is cloned into a mammalian expression vector. The mammalian expression vector is used to transform a mammalian cell line to produce a recombinant mammalian cell line. The recombinant mammalian cell line is cultivated under conditions that permit expression of the modified receptor. The recombinant mammalian cell line or membranes isolated from the recombinant mammalian cell line are used in assays to identify compounds that bind to the modified receptor.

EXAMPLE 15

Screening Assay

Recombinant cells containing DNA encoding a modified receptor, membranes derived from the recombinant cells, or recombinant modified receptor preparations derived from the cells or membranes may be used to identify compounds that modulate modified G-protein coupled receptor activity. Modulation of such activity may occur at the level of DNA, RNA, protein or combinations thereof. One method of identifying compounds that modulate modified G-protein coupled receptor, comprises:

(a) mixing a test compound with a solution containing modified G-protein coupled receptor to form a mixture;

(b) measuring modified G-protein coupled receptor activity in the mixture; and

(c) comparing the modified G-protein coupled receptor activity of the mixture to a standard.

WHAT IS CLAIMED IS:

1. Isolated DNA encoding a modified receptor, the modified receptor being derived from a G-protein coupled receptor having seven transmembrane domains and the modified receptor having deletions in the third intracellular domain, or a functional derivative thereof.

2. The DNA of Claim 1 wherein the modified receptor is a modified β3-adrenergic receptor.

3. Isolated RNA encoded by the isolated DNA of Claim 1 or its complementary sequence.

4. Isolated RNA encoded by the isolated DNA of Claim 2 or its complementary sequence.

5. An expression vector containing the isolated DNA of Claim 1.

6. A recombinant host cell containing the expression vector of Claim 5.

7. A process for the production of a modified G-protein coupled receptor, comprising:

a) transforming a host cell with the isolated DNA of Claim 1 to produce a recombinant host cell;

b) culturing the recombinant host cell under conditions which allow the production of modified G-protein coupled receptor; and

c) recovering the modified G-protein coupled receptor.

8. The modified G-protein coupled receptor produced by the process of Claim 7.

9. The process of Claim 7 wherein the modified G-protein coupled receptor is a modified beta-3 adrenergic receptor.

10. An isolated and purified modified G-protein coupled receptor, the receptor having seven transmembrane domains and having amino acids deleted from the third transmembrane domain, or a functional derivative thereof.

11. The purified modified G-protein coupled receptor of Claim 10 which is a modified beta-3 adrenergic receptor.

12. A method of identifying compounds that modulate modified G-protein coupled receptor activity, comprising:

(a) mixing a test compound with a solution containing modified G-protein coupled receptor to form a mixture;

(b) measuring modified G-protein coupled receptor activity in the mixture; and

(c) comparing the modified G-protein coupled receptor activity of the mixture to a standard.

13. Compounds identified by the method of Claim 12.

14. Pharmaceutical compositions comprising the compound of Claim 13.

15. A method for identifying compounds which specifically bind to a modified G-protein coupled receptor, comprising:

(a) cloning a G-protein coupled receptor;

(b) altering the DNA sequence encoding the third intracellular domain of the cloned G-protein coupled receptor;

(c) splicing the altered receptor into an expression vector to form a construct;

(d) introducing the construct into a cell which does not express the altered receptor in the absence of the introduced construct;

(e) incubating cells or membranes isolated from cells produced in step c with a quantifiable compound known to bind to the receptor; and

(f) adding test compounds so as to compete the quantifiable compound from the receptor.

16. Compounds identified by the method of Claim 15.

17. A method of making a modified G-protein coupled receptor, comprising:

(a) isolating DNA encoding a G-protein coupled receptor;

(b) altering the DNA of step (a) by deleting at least one nucleotide from DNA encoding the third intracellular domain of the G-protein coupled receptor;

(c) isolating the altered DNA;

(d) expressing the altered DNA; and

(e) recovering the modified G-protein coupled receptor.

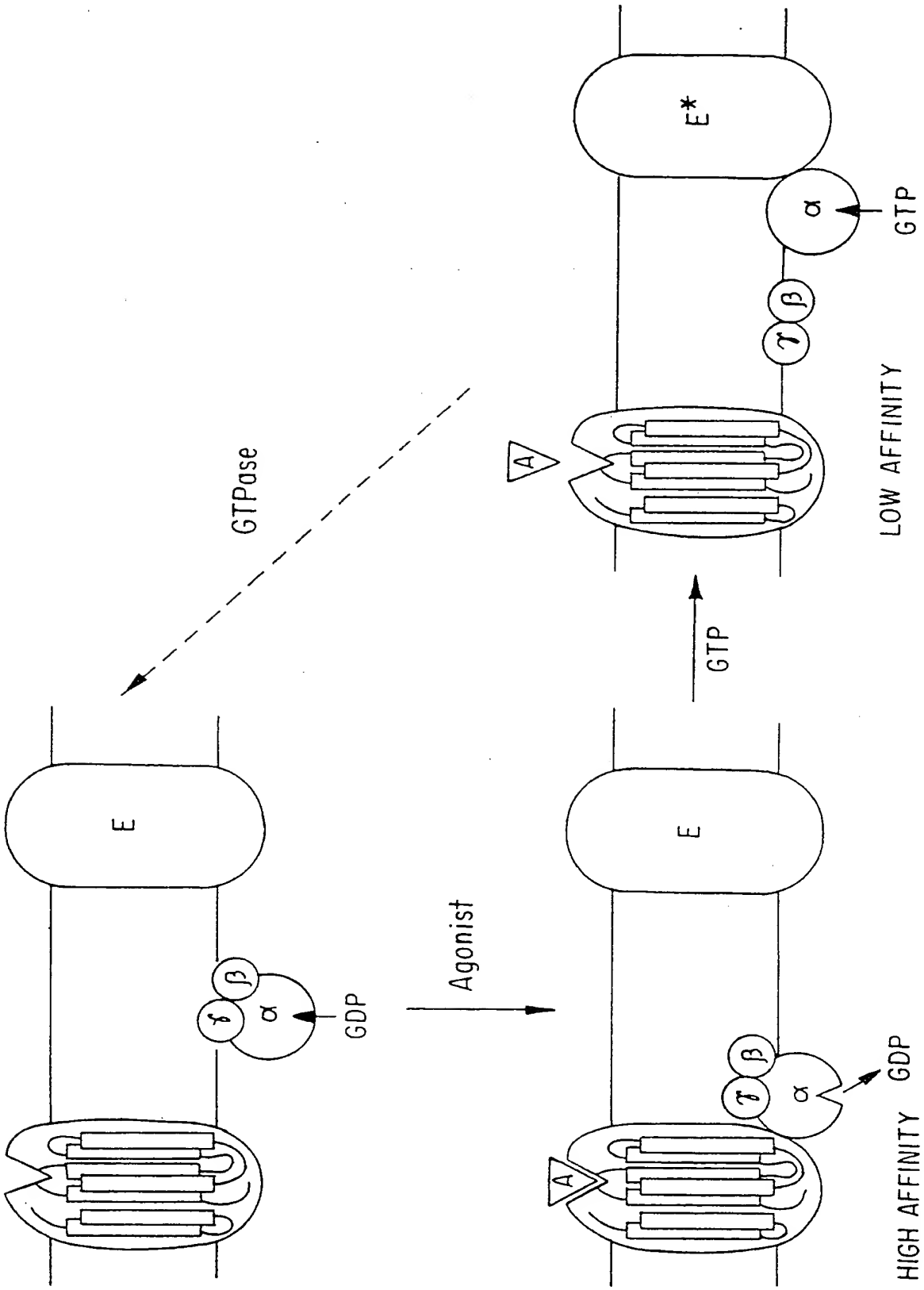
18. The modified G-coupled protein receptors of Claim 17.

19. The method of Claim 17 wherein between six and thirteen nucleotides are deleted from DNA encoding the third intracellular domain of the G-protein coupled receptor.

20. The isolated DNA of Claim 1 wherein the modified G-protein coupled receptor is selected from the group consisting of:

(a) D(277-289) beta-3 adrenergic receptor; and
(b) D(227-234) beta-3 adrenergic receptor.

21. The isolated and purified receptor of Claim 10 wherein the modified beta adrenergic receptor is selected from the group consisting of D(277-289) beta-3 adrenergic receptor and D(227-234) beta-3 adrenergic receptor.


E*
α
GTP
β
γ
A
LOW AFFINITY
GTPase
GTP
E
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β
γ
α
GDP
Agonist
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γ
α
GDP
A
HIGH AFFINITY

FIG. 1

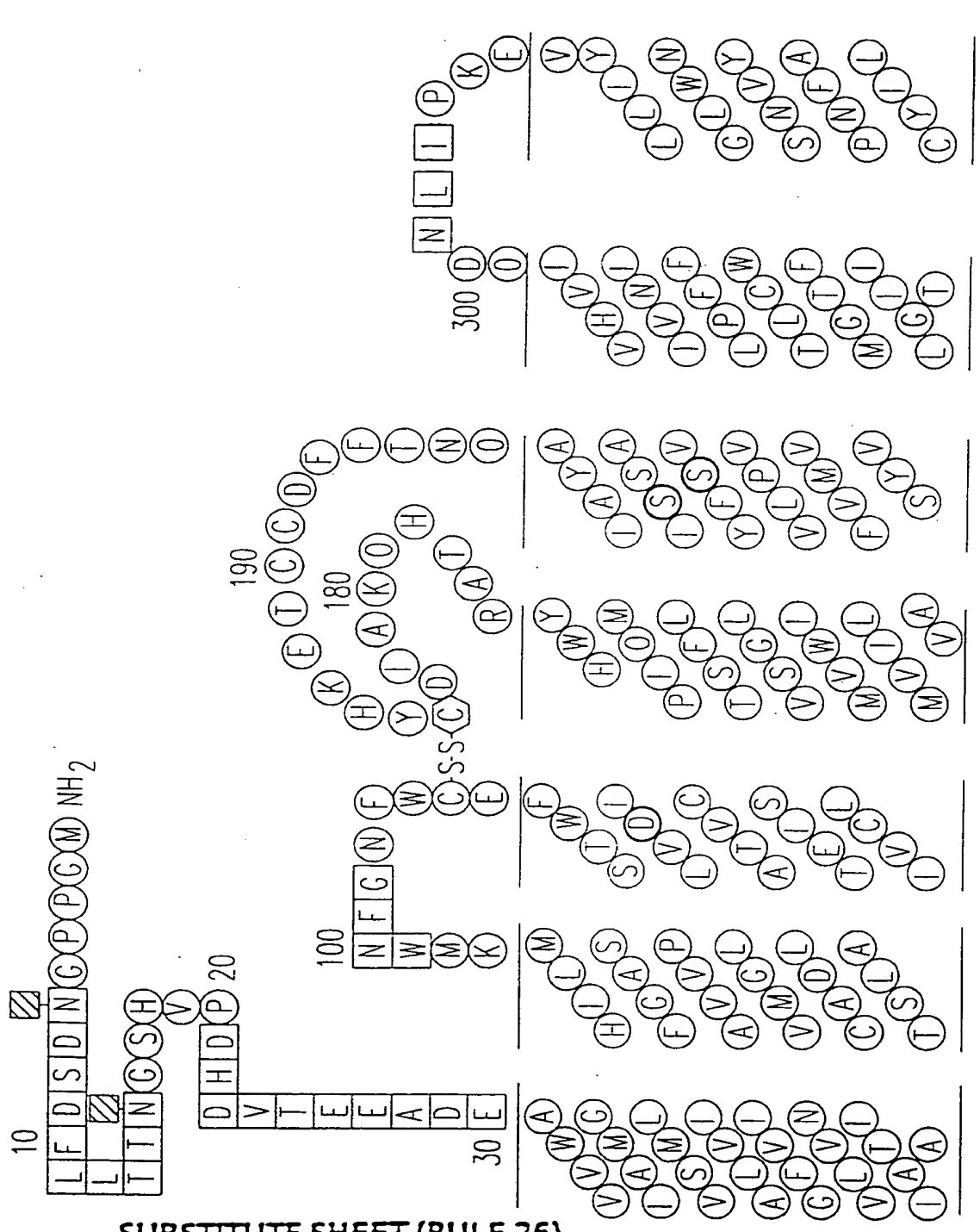

FIG.2A

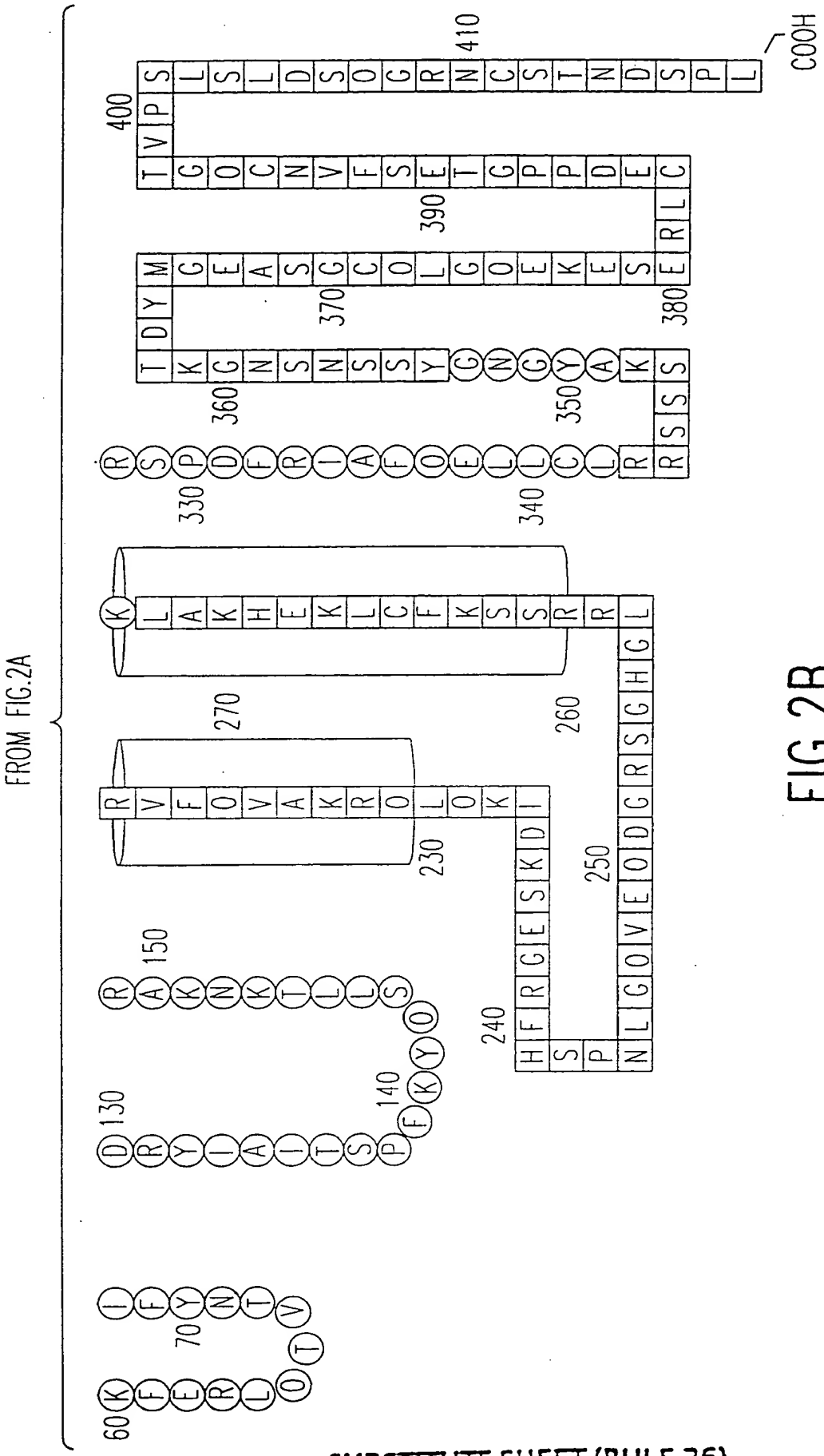

FIG.2B

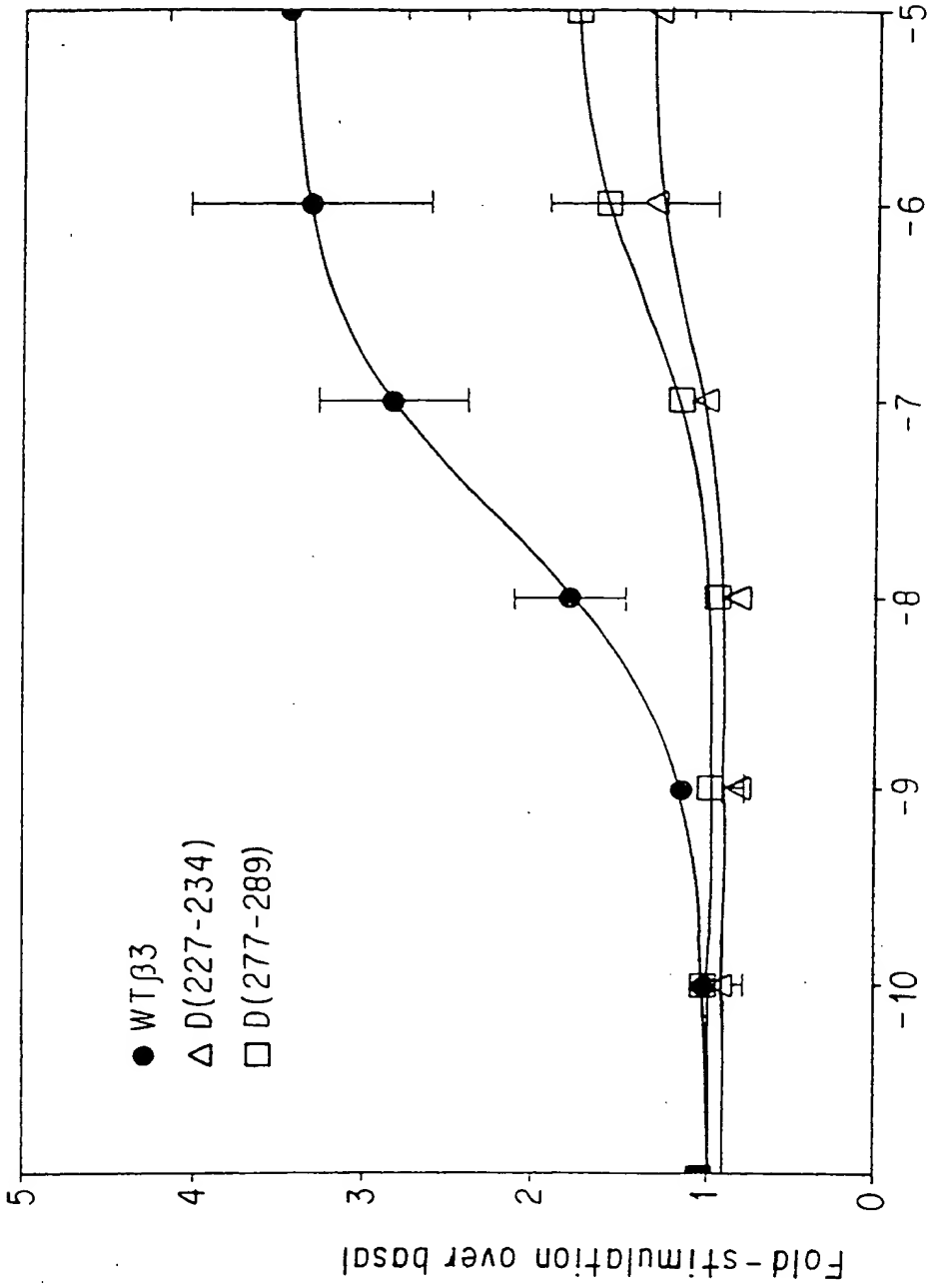

FIG. 3

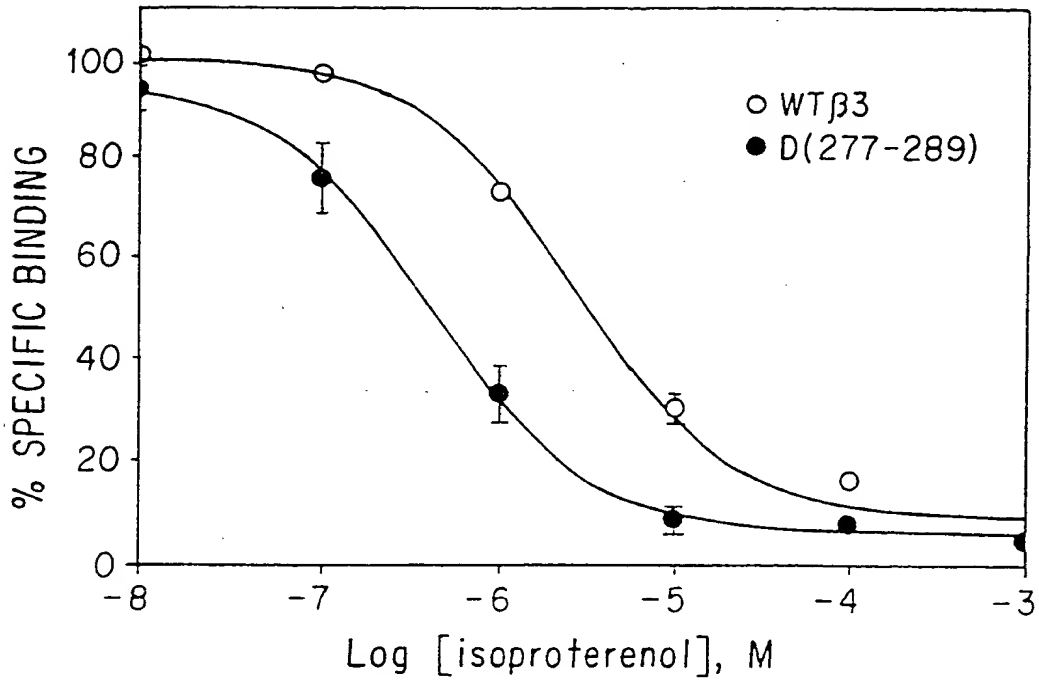

FIG. 4A

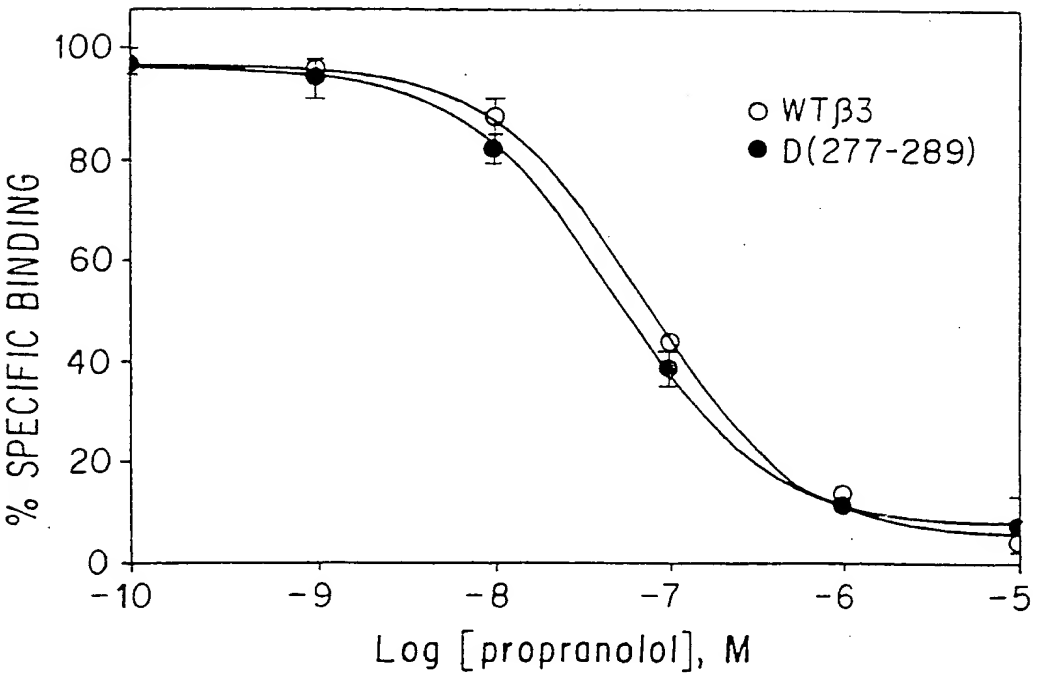

FIG. 4B

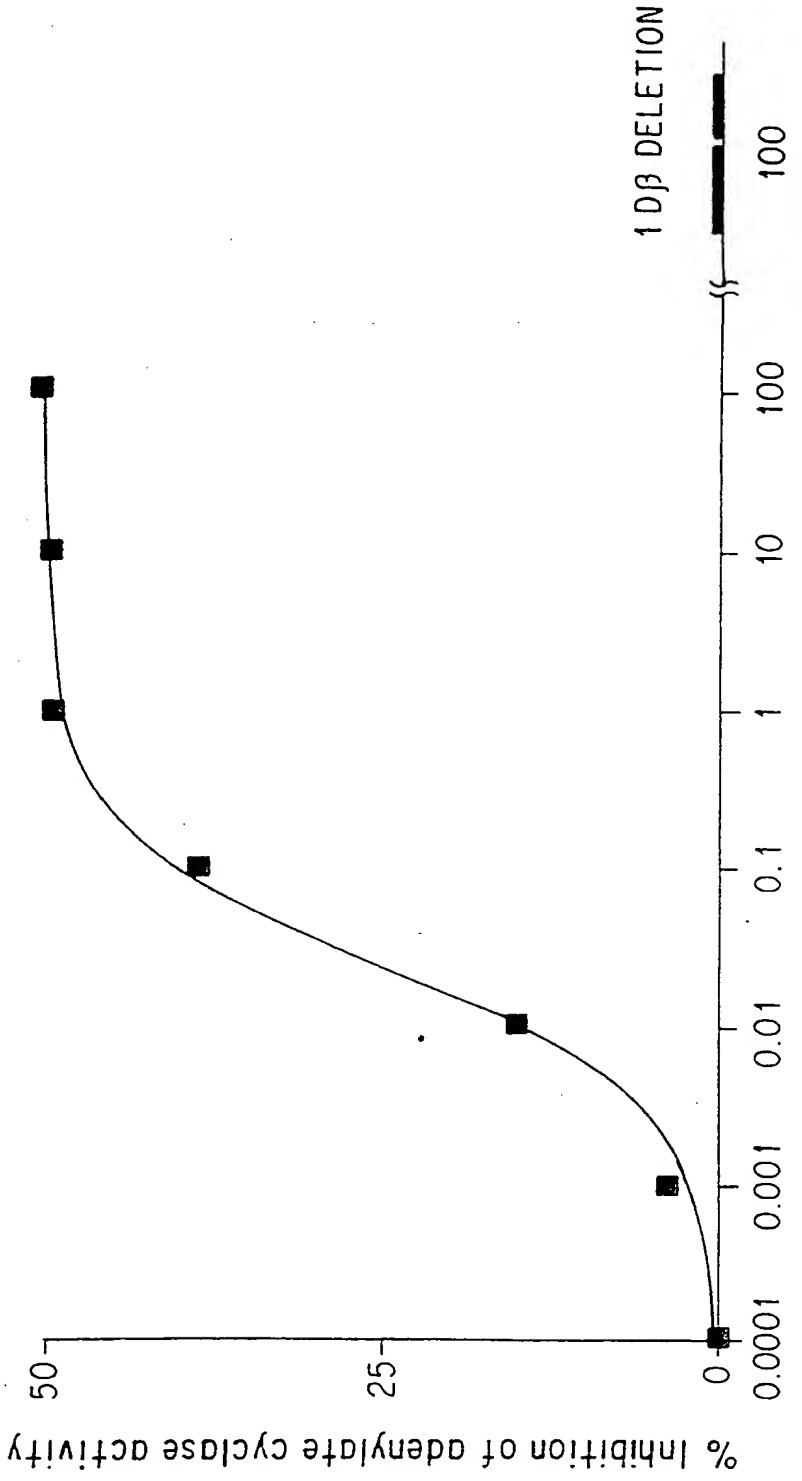

FIG. 5

PROPERTIES OF MUTANT β2 RECEPTORS

Mutant	Kd (nM)				Adenylyl cyclase activation	
	-Gpp(NH)p			+Gpp(NH)p		
	K_H	K_L	%H	Kd	Kact (nM)	fold stimulation
Wild type βAR	3	200	70	200	15	3.2
D(222-229)βAR	6	—	100	7	—	1.0
D(258-270)βAR	8	—	100	10	—	1.3

FIG. 6

	Binding affinity (M)				cAMP production	
	K_H	$\%K_H$	K_L	$\%K_L$	Max stimulation (fold over basal)	Kact (M)
WT β3AR	$5.0 \pm 2.1 \times 10^{-8}$	28±4	$2.6 \pm 0.7 \times 10^{-6}$	72±4	3.5±0.7	$2.6 \pm 0.3 \times 10^{-8}$
D227-234	$1.8 \pm 1.0 \times 10^{-7}$	100	n.d		1.3±0.4	n.d
D277-289	$2.2 \pm 0.4 \times 10^{-7}$	87±13	4.4×10^{-6}*	13±13	1.8±0.7	n.d

n.d. not detectable.
*The low affinity binding site was detected in only one experiment.

FIG. 7

Radialigand binding properties of mutant 5HT1Dβ receptors				
Receptor	Specific Binding (dpm)	Specific Binding (+GppNHp) (dpm)	% Guanine nucleotide Shift	% Adenylate Cyclase Inhibition
Wild Type	4120	1553	67	45.6
D(231-239)1Dβ Clone1	2046	1885	8	-4.9
D(231-239)1Dβ Clone21	716	710	1	-3.3
D(231-239)1Dβ Clone65	1450	1292	11	0.3

FIG. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06900

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1; 530/350; 435/7.1, 7.2, 69.1, 172.1, 240.1, 252.3, 254.11, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Medline, Dialog, WPI
search terms: β3-Adrenergic Receptor, modified, variant, deletions in the third intracellular domain

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Molecular Pharmacology, Volume 37, Number 6, issued June 1990, Cheung et al, "Separation of the Structural Requirements for Agonist-Promoted Activation and Sequestration of the β-Adrenergic Receptor", pages 775-779, see pages 776-777.	1-21
Y	Science, Volume 245, issued 08 September 1989, Emorine et al, "Molecular Characterization of the Human β_3-Adrenergic Receptor", pages 1118-1121, see entire document.	1-21

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 AUGUST 1995	15SEP1995
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer Sally P. Teng
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06900

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Molecular Pharmacology, Volume 38, Number 3, issued September 1990, Johnson et al, "Identification of a Specific Domain in the β-Adrenergic Receptor Required for Phorbol Ester-Induced Inhibition of Catecholamine-Stimulated Adenylyl Cyclase", pages 289-293, see pages 292-293.	1-21
Y	Molecular Pharmacology, Volume 40, issued 1991, Granneman et al, "Molecular Cloning and Expression of the Rat β_3-Adrenergic Receptor", pages 895-899, see pages 896-898.	1-21
Y	The Embo Journal, Volume 10, Number 12, issued 1991, Nahmias et al, "Molecular Characterization of the Mouse β3-Adrenergic Receptor: Relationship with the Atypical Receptor of Adipocytes", pages 3721-3727, see pages 3722-3724.	1-21
Y	Molecular Pharmacology, Volume 44, issued September 1993, Proll et al, "β_2-Adrenergic Receptor Mutants Reveal Structural Requirements for the Desensitization Observed with Long Term Epinephrine Treatment", pages 569-574, see pages 570-574.	1-21

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/06900

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6): C07K 14/705; C07H 21/00; C12N 5/10, 15/63, 15/70, 15/79; C12Q 1/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL : 536/23.1; 530/350; 435/7.1, 7.2, 69.1, 172.1, 240.1, 252.3, 254.11, 320.1

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